APPENDIX I

LAB VISION CORPORATION | NEOMARKERS

RESEARCH USE ONLY DATA SHEET

Rev 022502I

Clinical customers please refer to IVD / ASR Data Sheet

Epidermal Growth Factor Receptor (EGFR) Ab-10 (Clone 111.6)

Mouse Monoclonal Antibody

Cat. #MS-378-P0, -P1, or -P (0.1ml, 0.5ml, or 1.0ml at 200µg/ml) (Purified Ab with BSA and Azide)
Cat. #MS-378-P1ABX or -PABX (0.1ml or 0.2ml at 1.0mg/ml) (Purified Ab without BSA and Azide)
Cat. #MS-378-B0, -B1, or -B (0.1ml, 0.5ml, or 1.0ml at 200µg/ml) (Biotin-Labeled Ab with BSA and Azide)
Cat. #MS-378-R7 (7.0ml) (Ready-to-Use for Immunohistochemical Staining)
Cat. #MS-378-PCS (5 Slides) (Positive Control for Histology)
Cat. #MS-378-PCL (0.1ml) (Positive Control for Western Blot)

Description: EGFR is type I receptor tyrosine kinase with sequence homology to erbB-1, -2, -3 -4 or HER-1, -2, -3 -4. It binds to Epidermal Growth Factor (EGF), Transforming Growth Factor-a (TGF-a), Heparin-binding EGF (HB-EGF), amphiregulin, betacellulin and epiregulin. EGFR is overexpressed in tumors of breast, brain, bladder, lung, gastric, head & neck, esophagus, cervix, vulva, ovary, and endometrium. It is predominantly present in squamous cell carcinomas.

Comments: Ab-10 is excellent for staining of routine formalin/paraffin tissues. It blocks the binding of EGF to EGFR.

Mol. Wt. of Antigen: 170kDa (wild type) and 145kDa (vIII variant)

Epitope: Extracellular domain

Species Reactivity: Human. Not mouse and rat.

Clone Designation: 111.6

Ig Isotype: IgG_1

Immunogen: Extracellular domain of human recombinant EGFR protein.

Applications and Suggested Dilutions:

- Effect on EGF Binding (Inhibitory)
  (Order Ab without sodium azide)
- Immunoprecipitation (Native only)
  (Use Protein G; Ab at 2µg/mg protein lysate)
- Western Blotting (Ab-12 is better)
  (Ab 2-4µg/ml for 2hrs at RT)
- Immunohistology (Formalin/paraffin)
  (Ab 2-4µg/ml for 30 min at RT)

* [Staining of formalin-fixed tissues REQUIRES digestion with Protease XXV at 1mg/ml PBS for 5 minutes at 37°C (***NEOMARKERS'*** Cat. #AP-9006)]

The optimal dilution for a specific application should be determined by the investigator.

Cellular Localization: Cell membrane

Positive Control: A431 cells. Skin, placenta or squamous cell carcinoma.

Supplied As: 200µg/ml antibody purified from the ascites fluid by Protein G chromatography. Prepared in 10mM PBS, pH 7.4, with 0.2% BSA and 0.09% sodium azide. Also available without BSA and azide at 1mg/ml, or

Prediluted antibody which is ready-to-use for staining of formalin-fixed, paraffin-embedded tissues.

Storage and Stability:

Ab with sodium azide is stable for 24 months when stored at 2-8°C. Antibody WITHOUT sodium azide is stable for 36 months when stored at below 0°C.

Suggested References:

1. Kawamoto T, *et. al.* Proceedings of the National Academy of Sciences, USA. 1983; 80:1337-41.
2. Sato JD, *et. al.* Mol Biol Med. 1983; 1(5):511-29.
3. den Hartigh JC, *et. al.* Biochimica Biophys Acta. 1993. 1148:249-56.

Limitations and Warranty:

Material Safety Data:

This product is not licensed or approved for administration to humans or to animals other than the experimental animals. Standard Laboratory Practices should be followed when handling this material. The chemical, physical, and toxicological properties of this material have not been thoroughly investigated. Appropriate measures should be taken to avoid skin and eye contact, inhalation, and ingestion. The material contains 0.09% sodium azide as a preservative. Although the quantity of azide is very small, appropriate care should be taken when handling this material as indicated above. The National Institute of Occupational Safety and Health has issued a bulletin citing the potential explosion hazard due to the reaction of sodium azide with copper, lead, brass, or solder in the plumbing systems. Sodium azide forms hydrazoic acid in acidic conditions and should be discarded in a

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RESEARCH USE ONLY DATA SHEET

Rev 022502I

Clinical customers please refer to IVD / ASR Data Sheet

Epidermal Growth Factor Receptor (EGFR) Ab-10 (Clone 111.6)

Mouse Monoclonal Antibody

Cat. #MS-378-P0, -P1, or -P (0.1ml, 0.5ml, or 1.0ml at 200µg/ml) (Purified Ab with BSA and Azide)
Cat. #MS-378-P1ABX or -PABX (0.1ml or 0.2ml at 1.0mg/ml) (Purified Ab without BSA and Azide)
Cat. #MS-378-B0, -B1, or -B (0.1ml, 0.5ml, or 1.0ml at 200µg/ml) (Biotin-Labeled Ab with BSA and Azide)
Cat. #MS-378-R7 (7.0ml) (Ready-to-Use for Immunohistochemical Staining)
Cat. #MS-378-PCS (5 Slides) (Positive Control for Histology)
Cat. #MS-378-PCL (0.1ml) (Positive Control for Western Blot)

large volume of running water to avoid deposits forming in metal drainage pipes.

For Research Use Only

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LAB VISION CORPORATION

Human Epidermal Growth Factor Receptor phosphoTyrosine 1068 ELISA EGFR [pY^{1068}]

Catalog No.:	**KHR9081 (one plate kit; 96 determinations)**
Sensitivity:	<0.3 units/mL
Range:	1.6 – 100 units/mL
Incubation Time:	4 hours
Simple:	Pre-coated strip-well plates Liquid stable chromogen and stop reagents Sample size: 100 µL
Economical:	One 96-well plate, plus reagents for 96 determinations Reagents are stable for multiple runs
Sample type:	Cell extracts, tissue homogenates, buffered solutions

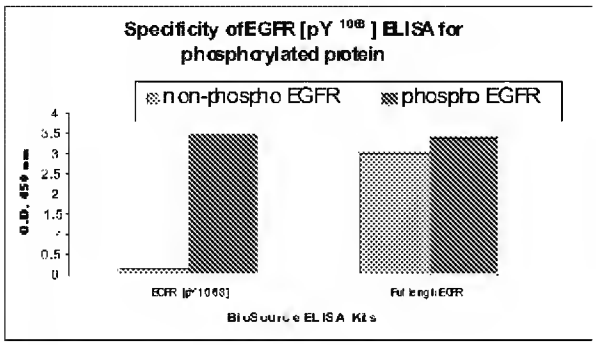
Human EGFR [pY^{1068}] ELISA Kit Summary Protocol

1. Add standards, specimens or controls to appropriate wells. Cells extracted in buffer containing SDS or other strong chemicals should be diluted in Standard Diluent 1:10 or greater prior to assay.
2. Incubate wells at room temperature for 2 hours.
3. Aspirate and wash wells 4x with Working Wash Buffer.
4. Add 100 µL Rabbit anti-EGFR [pY^{1068}] PSSA to each well.
5. Incubate wells at room temperature for 1 hour.
6. Aspirate and wash wells 4x with Working Wash Buffer.
7. Add 100 µL anti-rabbit IgG HRP Working Conjugate to each well.
8. Incubate for 0.5 hour at room temperature.
9. Aspirate and wash wells 4x with Working Wash Buffer.
10. Add 100 µL of Stabilized Chromogen to each well.
11. Incubate at room temperature for 30 minutes in the dark.
12. Add 100 µL of Stop Solution to each well.
13. Read absorbance at 450 nm.

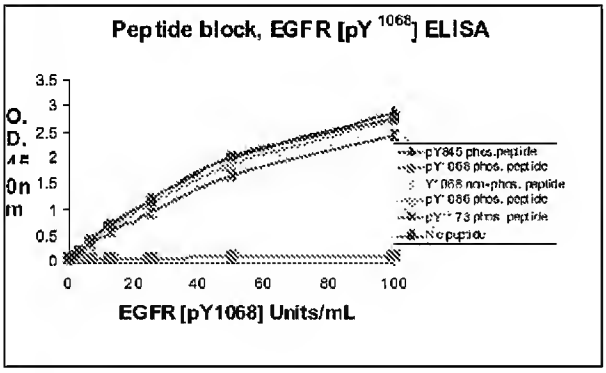
Performance Data

Sensitivity: The analytical sensitivity is <0.3 units/mL, determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times. 1 unit is defined as the amount of EGFR [pY^{1068}] that results from autophosphorylation of 42 pg EGFR purified from A431 cells.

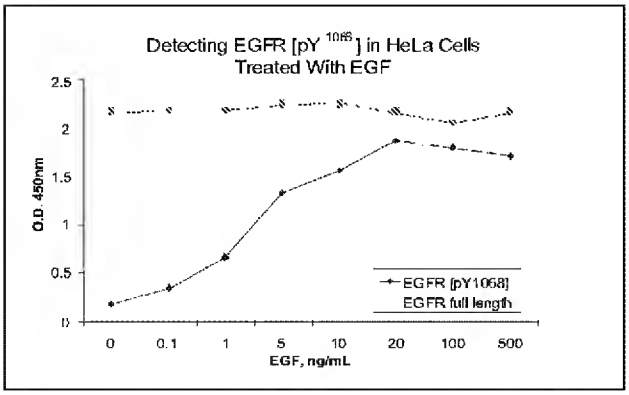
Specificity:



This EGFR [pY^{1068}] ELISA kit is specific for measurement of phosphorylated human EGFR at tyrosine 1068. The kit does not detect non-phosphorylated EGFR protein.



Specificity is confirmed by peptide competition. Data show that only the blocking peptide corresponding to the region surrounding tyrosine 1068, containing the phospho-tyrosine, could block the ELISA signal. The same sequence containing non-phosphorylated tyrosine at position 1068 did not block the signal.



HeLa cells were treated with EGF at varying concentrations. Levels of EGFR [pY^{1068}] increased with EGF treatment concentration, while full length EGFR remained constant. Data show that levels of EGFR [pY^{1068}] phosphorylation are dependent on levels of EGF.

Rabbit (polyclonal) Anti-Epidermal Growth Factor Receptor [pY^{1086}] Phosphospecific Antibody, Unconjugated

PRODUCT ANALYSIS SHEET

Catalog Number: 44-790G (10 mini-blot size)

Lot Number: 0401

Volume: 100 µL

Form of Antibody: Rabbit polyclonal immunoglobulin in Dulbecco's phosphate buffered saline (without Mg^{2+} and Ca^{2+}), pH 7.3 (+/- 0.1), 50% glycerol with 1.0 mg/mL BSA (IgG, protease free) as a carrier.

Preservative: 0.05% sodium azide (Caution: sodium azide is a poisonous and hazardous substance. Handle with care and dispose of properly.)

Purification: Purified from rabbit serum by sequential epitope-specific chromatography. The antibody has been negatively preadsorbed using a non-phosphopeptide corresponding to the site of phosphorylation to remove antibody that is reactive with non-phosphorylated EGFR. The final product is generated by affinity chromatography using an EGFR-derived peptide that is phosphorylated at tyrosine 1086.

Immunogen: The antiserum was produced against a chemically synthesized phosphopeptide derived from the region of human EGFR that contains tyrosine 1086. The sequence is conserved in mouse and rat.

Target Summary: Epidermal Growth Factor Receptor, also known as ErbB-1 (EGFR, a 185 kDa glycoprotein) is a transmembrane tyrosine kinase that regulates a variety of biological responses ranging from mitogenesis to stress signaling. The EGFR consists of a large extracellular domain, a single transmembrane domain and a cytoplasmic domain that exhibits kinase activity. Upon binding of EGF to the extracellular domain, the receptor undergoes dimerization and becomes phosphorylated on several tyrosine residues within the cytoplasmic domain, one of which is tyrosine 1086. This results in EGFR activation and increased tyrosine kinase activity toward a variety of intracellular substrates.

Reactivity: Human EGFR. Mouse and rat (100% homologous) have not been tested, but are expected to react.

Applications: The antibody has been used in Western blotting. Previous lots of this antibody have been used in immunostaining.

Suggested Working Dilutions: For Western blotting applications, we recommend using the antibody at a 1:1000 starting dilution. The optimal antibody concentration should be determined empirically for each specific application.

Storage: Store at −20°C. We recommend a brief centrifugation before opening to settle vial contents. Then, apportion into working aliquots and store at −20°C. For shipment or short-term storage (up to one week), 2-8°C is sufficient.

Positive Controls Used: Human epidermoid carcinoma (A431) cells +/- EGF.

Related Products:

Antibodies:

EGFR Sample Pack Cat. # 44-799G

ERK1&2 [pTpY$^{185/187}$], Cat. # 44-680G

EGFR [pY^{992}], Cat. # 44-786G

ELISAs: EGFR [pY^{1068}], Cat. # KHR9081

EGFR [pY^{1173}], Cat. # KHR9071

Extracts: A431 cell extracts +/- EGF, Cat. # 55-130

References:

Cuadrado, A., et al. (2003) Aplidin induces apoptosis in human cancer cells via glutathione depletion and sustained activation of the epidermal growth factor receptor, Src, JNK, and p38 MAPK. J. Biol. Chem. 278(1):241-250 (cites the use of cat. # 44-788G, 44-790G, 44-792G and 44-794G).

Piiper, A., et al. (2002) Cyclic AMP induces transactivation of the receptors for epidermal growth factor and nerve growth factor, thereby modulating activation of MAP kinase, Akt, and neurite outgrowth in PC12 cells. J. Biol. Chem. 277(46):43623-43630 (cites the use of this antibody).

Miljan, E.A., et al. (2002) Interaction of the extracellular domain of the epidermal growth factor receptor with gangliosides. J. Biol. Chem. 277(12):10108-10113 (cites the use of this antibody).

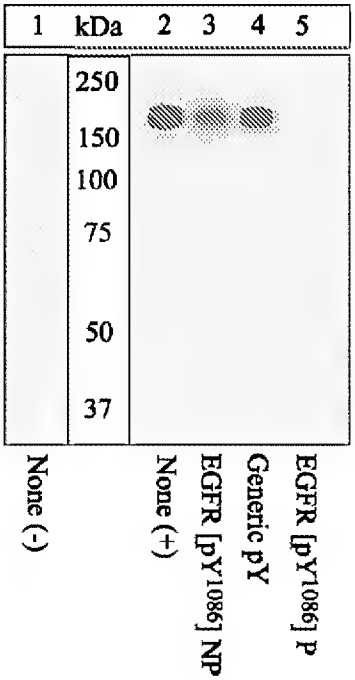
Moro, L., et al. (2002) Integrin-induced epidermal growth factor (EGF) receptor activation requires c-Src and p130Cas and leads to phosphorylation of specific EGF receptor tyrosines. J. Biol. Chem. 277(11):9405-9414 (cites the use of cat. # 44-784G, 44-786G, 44-790G, 44-792G and 44-794G).

Janne, P.A., et al. (2002) Inhibition of epidermal growth factor receptor signaling in malignant pleural mesothelioma. Cancer Res. 62(18):5242-5247 (cites the use of cat. # 44-680G and 44-790G).

Ushio-Fukai, M., et al. (2001) Epidermal growth factor receptor transactivation by angiotensin II requires reactive oxygen species in vascular smooth muscle cells. Arterioscler. Thromb. Vasc. Biol. 21(4):489-495 (cites the use of cat. # 44-660G, 44-786G, 44-788G, 44-790G, 44-792G and 44-794G).

Sieg, D.J., et al. (2000) FAK integrates growth-factor and integrin signals to promote cell migration. Nat. Cell Biol. 2(5):249-256.

Keilhack, H., et al. (1998) Phosphotyrosine 1086 mediates binding of the protein-tyrosine phosphatase SHP-1 to the epidermal growth factor receptor and attenuation of receptor signaling. J. Biol. Chem. 273(38):24839-24846.



Peptide Competition

Extracts of A431 cells unstimulated (1) or stimulated with 200 ng/mL EGF for 15 minutes (2-5) were resolved by SDS-PAGE on a 10% Tris-glycine gel and transferred to PVDF. The membrane was blocked with a 5% BSA-TBST buffer overnight at 4°C, then incubated with the EGFR [pY^{1086}] antibody for two hours at room temperature in a 1% BSA-TBST buffer, following prior incubation with: no peptide (1), the non-phosphopeptide corresponding to the phosphopeptide immunogen (2), a generic phosphotyrosine-containing peptide (3), or the phosphopeptide immunogen (4). After washing, the membrane was incubated with goat F(ab')$_2$ anti-rabbit IgG HRP conjugate (Cat. # ALI4404) and signals were detected using the Pierce SuperSignal™ method.

The data show that only the phosphopeptide corresponding to EGFR [pY^{1086}] blocks the antibody signal, demonstrating the specificity of the antibody. The data also show the induction of EGFR [pY^{1086}] phosphorylation by the addition of EGF to this cell system

Western Blotting Procedure

1. Lyse approximately 10^7 cells in 0.5 mL of ice cold Cell Lysis Buffer (formulation provided below). This buffer, a modified RIPA buffer, is suitable for recovery of most proteins, including membrane receptors, cytoskeletal-associated proteins, and soluble proteins. This cell lysis buffer formulation is available as a separate product which requires supplementation with protease inhibitors immediately prior to use (Cat. # FNN0011). Other cell lysis buffer formulations, such as Laemmli sample buffer and Triton-X 100 buffer, are also compatible with this procedure. Additional optimization of the cell stimulation protocol and cell lysis procedure may be required for each specific application.
2. Remove the cellular debris by centrifuging the lysates at 14 000 x g for 10 minutes. Alternatively lysates may be ultracentrifuged at 100,000 x g for 30 minutes for greater clarification.
3. Carefully decant the clarified cell lysates into clean tubes and determine the protein concentration using a suitable method, such as the Bradford assay. Polypropylene tubes are recommended for storing cell lysates.
4. React an aliquot of the lysate with an equal volume of 2x Laemmli Sample Buffer (125 mM Tris, pH 6.8, 10% glycerol, 10% SDS, 0.006% bromophenol blue, and 130 mM dithiothreitol [DTT]) and boil the mixture for 90 seconds at 100°C.
5. Load 10-30 µg of the cell lysate into the wells of an appropriate single percentage or gradient minigel and resolve the proteins by SDS-PAGE.
6. In preparation for the Western transfer, cut a piece of PVDF slightly larger than the gel. Soak the membrane in methanol for 1 minute, then rinse with ddH_2O for 5 minutes. Alternatively, nitrocellulose may be used.
7. Soak the membrane, 2 pieces of Whatman paper, and Western apparatus sponges in transfer buffer (formulation provided below) for 2 minutes.
8. Assemble the gel and membrane into the sandwich apparatus.
9. Transfer the proteins at 140 mA for 60-90 minutes at room temperature.
10. Following the transfer, rinse the membrane with Tris buffered saline for 2 minutes.
11. Block the membrane with blocking buffer (formulation provided below) overnight at 4°C or for one hour at room temperature.
12. Incubate the blocked blot with primary antibody at a 1:1000 starting dilution in Tris buffered saline supplemented with 1% Ig-free BSA and 0.1% Tween-20 overnight at 4°C or for two hours at room temperature.
13. Wash the blot with several changes of Tris buffered saline supplemented with 0.1% Tween 20.
14. Detect the antibody band using an appropriate secondary antibody, such as goat $F(ab')_2$ anti-rabbit IgG alkaline phosphatase conjugate (Cat. # ALI4405) or goat $F(ab')_2$ anti-rabbit IgG horseradish peroxidase conjugate (Cat. # ALI4404) in conjunction with your chemiluminescence reagents and instrumentation.

Cell Lysis Buffer
Formulation:
10 mM Tris, pH 7.4
100 mM NaCl
1 mM EDTA
1 mM EGTA
1 mM NaF
20 mM $Na_4P_2O_7$
2 mM Na_3VO_4
0.1% SDS
0.5% sodium deoxycholate
1% Triton-X 100
10% glycerol
1 mM PMSF (made from a 0.3 M stock in DMSO) or 1 mM AEBSF (water soluble version of PMSF)
60 µg/mL aprotinin
10 µg/mL leupeptin
1 µg/mL pepstatin
(alternatively, protease inhibitor cocktail such as Sigma Cat. # P2714 may be used)

Transfer Buffer
Formulation:
2.4 gm Tris base
14.2 gm glycine
200 mL methanol
Q.S. to 1 liter, then add
1 mL 10% SDS.
Cool to 4°C prior to use.

Tris Buffered Saline
Formulation:
20 mM Tris-HCl, pH 7.4
0.9% NaCl

Blocking Buffer
Formulation:
100 mL Tris buffered saline
5 gm Ig-free BSA
0.1 mL Tween 20

Peptide Competition Experiment

BioSource's Phosphorylation Site Specific Antibodies (PSSAs) have been developed to enable the specific and sensitive detection of phosphorylation of particular amino acid residues in target proteins, while circumventing the need for protein purification, phosphopeptide mapping or handling radioactivity. The specificity of a PSSA in each experimental system can be confirmed through peptide competition. In this technique, aliquots of antibody are pre-incubated with peptide containing the sequence of the phosphopeptide immunogen used to raise the PSSA and the corresponding non-phosphopeptide. Following preincubation with the peptide, each antibody preparation is then used as a probe in antibody-based detection methods, such as Western blotting, immunocytochemistry, flow cytometry, or ELISA. With a PSSA specific for the phosphorylated target protein, pre-incubation with an excess of peptide containing the sequence of the phosphopeptide immunogen will block all antigen binding sites, while pre-incubation with the corresponding non-phosphopeptide will not affect the antibody.

BioSource has developed a line of control peptides specifically for use in peptide competition experiments with our PSSAs. These peptides, available as separate BioSource catalog items, are provided in pairs which contain the sequences of the phosphopeptide immunogen and the corresponding non-phosphopeptide.

In performing the Peptide Competition Experiment, it is important to note that the optimal dilutions of both antibody and peptide should be determined empirically for each specific application. The optimal dilution of antibody in these procedures is below saturating, as determined by previous experiments in your system. If an optimal antibody dilution has not been determined in your system, please refer to the Suggested Working Dilution on the antibody Product Analysis Sheet for guidance on an appropriate starting dilution. The optimal dilution of peptide used in these procedures will depend on the overall affinity or avidity of the antibody, as well as the quantity of the target antigen. A 50-150 fold molar excess of peptide to antibody is found to be effective for most peptide competition experiments.

In the example presented below, the PSSA is used as a dilution of 1:1000 and the peptides are used at a concentration of 333 nM. The total volume of the phosphopeptide and non-phosphopeptide-pre-incubated antibody preparations is 2 mL, sufficient for probing Western blot strips, as well as for use in other antibody-based detection methods. Under these conditions, the molar excess of peptide to antibody is ≥50.

Procedure:

1. Prepare three *identical test samples*, such as identical PVDF or nitrocellulose strips to which the protein of interest has been transferred. The test samples should be blocked using a blocking buffer, such as Tris buffered saline supplemented with 0.1% Tween 20, and either 5% BSA or 5% non-fat dried milk.
2. Prepare 6.5 mL of *working antibody stock solution* (1:1000 in this example) by adding 6.5 µL of antibody stock solution to 6.5 mL of buffer containing blocking protein, such as TBS supplemented with 0.1% Tween 20, and either 3% BSA or 3% non-fat dried milk.
3. Apportion the unused PSSA into working aliquots and store at –20°C for future use (the stock PSSA contains 50% glycerol and will not freeze at this temperature).
4. Allow the *lyophilized control peptides* to reach room temperature, ideally under desiccation.
5. Reconstitute each of the control peptides (supplied at 0.1 mg/vial) to a concentration of 66.7 µM with nanopure water. For a peptide with a molecular mass of 1500 (stated on the peptide Product Analysis Sheet), reconstitution with 1 mL water yields a solution with a concentration of 66.7 µM.
6. Apportion the unused reconstituted peptide solutions into working aliquots and store at -20°C for future use.
7. Label 3 test tubes as follows:
   - tube 1: water only no peptide control
   - tube 2: phosphopeptide
   - tube 3: non-phosphopeptide
8. Into each tube, pipette the following components
   - tube 1: 2 mL diluted PSSA solution plus 10 µL nanopure water
   - tube 2: 2 mL diluted PSSA solution plus 10 µL phosphopeptide
   - tube 3: 2 mL diluted PSSA solution plus 10 µL non-phosphopeptide
9. Incubate the three tubes for 30 minutes at room temperature with gentle rocking. During this incubation, the peptides have the chance to bind to the combining site of the antibody.
10. At the end of the incubation step, transfer the contents of each of the three tubes to clean reaction vessels containing one of the three identical test samples.

    For Western blotting strips:
    - Incubate the strips with the pre-incubated antibody preparations for 1 hour at room temperature or overnight at 4°C.
    - Wash each strip four times, five minutes each, to remove unbound antibody.
    - Transfer each strip to a new solution containing a labeled secondary antibody [e.g., goat $F(ab')_2$ anti-rabbit IgG alkaline phosphatase conjugate (Cat. # ALI4405) or goat $F(ab')_2$ anti-rabbit IgG horseradish peroxidase conjugate (Cat. # ALI4404)].
    - Remove unbound secondary antibody by thorough washing, and develop the signal using your chemiluminescent reagents and instrumentation.

The signal obtained with antibody incubated with the "Water Only, No Peptide Control" (Tube 1), represents the maximum signal in the assay. This signal should be eliminated by pre-incubation with the "Phosphopeptide" (Tube 2), while pre-incubation with the "Non-Phosphopeptide" (Tube 3) should not impact the signal. If the "Phosphopeptide" only partially eliminates the signal, repeat the procedure using twice the volume of water or peptide solutions listed in Step 8. If partial competition is seen following pre-incubation with the "Non-Phosphopeptide" repeat the procedure using half the volumes of water or peptide solutions listed in Step 8.

Rabbit (polyclonal) Anti-Epidermal Growth Factor Receptor [pY^{1148}] Phosphospecific Antibody, Unconjugated

PRODUCT ANALYSIS SHEET

Catalog Number: 44-792G (10 mini-blot size)

Lot Number: 0401

Volume: 100 µL

Form of Antibody: Rabbit polyclonal immunoglobulin in Dulbecco's phosphate buffered saline (without Mg^{2+} and Ca^{2+}), pH 7.3 (+/- 0.1), 50% glycerol with 1.0 mg/mL BSA (IgG, protease free) as a carrier.

Preservative: 0.05% sodium azide (Caution: sodium azide is a poisonous and hazardous substance. Handle with care and dispose of properly.)

Purification: Purified from rabbit serum by sequential epitope-specific chromatography. The antibody has been negatively preadsorbed using (i) a non-phosphopeptide corresponding to the site of phosphorylation to remove antibody that is reactive with non-phosphorylated EGFR, and (ii) a generic tyrosine phosphorylated peptide to remove antibody that is reactive with phospho-tyrosine (irrespective of the sequence). The final product is generated by affinity chromatography using an EGFR-derived peptide that is phosphorylated at tyrosine 1148.

Immunogen: The antiserum was produced against a chemically synthesized phosphopeptide derived from the region of EGFR that contains tyrosine 1148. The sequence is conserved in mouse, rat and chicken.

Target Summary: Epidermal Growth Factor Receptor, also known as ErbB-1 (EGFR, a 185 kDa glycoprotein) is a transmembrane tyrosine kinase that regulates a variety of biological responses ranging from mitogenesis to stress signaling. The EGFR consists of a large extracellular domain, a single transmembrane domain and a cytoplasmic domain that exhibits kinase activity. Upon binding of EGF to the extracellular domain, the receptor undergoes dimerization and becomes phosphorylated on several tyrosine residues within the cytoplasmic domain, one of which is tyrosine 1148. This results in EGFR activation and increased tyrosine kinase activity toward a variety of intracellular substrates.

Reactivity: Human EGFR. Mouse, rat and chicken (100% homologous) EGFR have not been tested, but are expected to react.

Applications: The antibody has been used in Western blotting.

Suggested Working Dilutions: For Western blotting applications, we recommend using the antibody at a 1:1000 starting dilution. The optimal antibody concentration should be determined empirically for each specific application.

Storage: Store at –20°C. We recommend a brief centrifugation before opening to settle vial contents. Then, apportion into working aliquots and store at –20°C. For shipment or short-term storage (up to one week), 2-8°C is sufficient.

Expiration Date: Expires one year from date of receipt when stored as instructed.

Positive Controls Used: A431 +/- EGF

Related Products:

Antibodies:

Akt/PKB [pT^{308}], Cat. # 44-602G

Akt/PKB [pS^{473}] monoclonal, Cat. # 44-621G

EGFR [pY845], Cat. # 44-784G

EGFR [pY1086], Cat. # 44-790G

EGFR [pY1173], Cat. # 44-794G

EGFR [R19/48] monoclonal, Cat. # 44-796G

EGFR [H9B4] monoclonal, Cat. # 44-798G

Related Products: (Continued)

Antibodies (continued):

EGFR [pY992], Cat. # 44-786G

EGFR [pY1068], Cat. # 44-788G

ERK1&2 [pTpY$^{185/187}$], Cat. # 44-680G

PLCγ-1 [pY^{783}], Cat. # 44-696G

EGFR Sampler Pack Cat. # 44-793G

ELISAs:

EGFR [pY^{1068}], Cat. # KHR9081

EGFR [pY^{1173}], Cat. # KHR9071

Extracts:

A431 +/- EGF, Cat. # 55-130

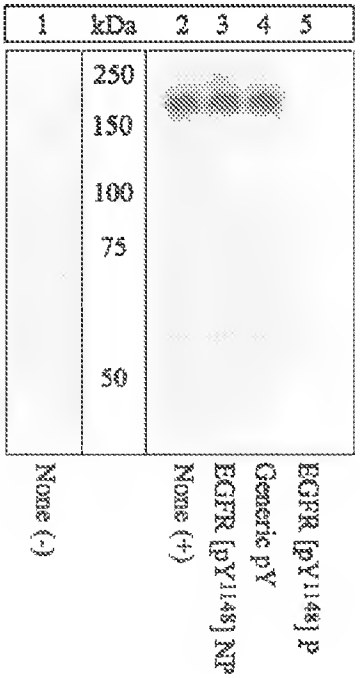
References:

Cuadrado, A., et al. (2003) Aplidin induces apoptosis in human cancer cells via glutathione depletion and sustained activation of the epidermal growth factor receptor, Src, JNK, and p38 MAPK. J. Biol. Chem. 278(1) 241-250 (cites the use of cat. # 44-788G, 44-790G, 44-792G and 44-794G).

Moro, L., et al. (2002) Integrin-induced epidermal growth factor (EGF) receptor activation requires c-Src and p130Cas and leads to phosphorylation of specific EGF receptor tyrosines. J. Biol. Chem. 277(11):9405-9414 (cites the use of cat. # 44-784G, 44-786G, 44-792G, 44-792G and 44-794G).

Ushio-Fukai, M., et al. (2001) Epidermal growth factor receptor transactivation by angiotensin II requires reactive oxygen species in vascular smooth muscle cells. Arterioscler. Thromb. Vasc. Biol. 21(4):489-495 (cites the use of cat. # 44-658 (discontinued), 44-660G, 44-786G, 44-788G, 44-790G, 44-792G and 44-794G).

Sieg, D.J., et al. (2000) FAK integrates growth-factor and integrin signals to promote cell migration. Nat. Cell Biol. 2(5):249-256.



Peptide Competition

Extracts of A431 cells unstimulated (1) or stimulated with 200 ng/mL EGF (2-5) for 15 minutes were resolved by SDS-PAGE on a 10% Tris-glycine gel and transferred to PVDF. The membrane was blocked with a 5% BSA-TBST buffer for one hour at room temperature, then incubated with the EGFR [pY^{1148}] antibody for two hours at room temperature in a 1% BSA-TBST buffer, following prior incubation with: no peptide (1, 2), non-phosphopeptide corresponding to the phosphopeptide immunogen (3), a generic phosphotyrosine-containing peptide (4), or the phosphopeptide immunogen (5). After washing, the membrane was incubated with goat F(ab')$_2$ anti-rabbit IgG HRP conjugate (Cat. # ALI4404) and signals were detected using the Pierce SuperSignal™ method.

The data show that only the phosphopeptide corresponding to EGFR [pY^{1148}] blocks the signal, demonstrating the specificity of the antibody. The data also show the induction of EGFR [pY^{1148}] phosphorylation by the addition of EGF to this cell system.

Western Blotting Procedure

1. Lyse approximately 10^7 cells in 0.5 mL of ice cold Cell Lysis Buffer (formulation provided below). This buffer, a modified RIPA buffer, is suitable for recovery of most proteins, including membrane receptors, cytoskeletal-associated proteins, and soluble proteins. This cell lysis buffer formulation is available as a separate product which requires supplementation with protease inhibitors immediately prior to use (Cat. # FNN0011). Other cell lysis buffer formulations, such as Laemmli sample buffer and Triton-X 100 buffer, are also compatible with this procedure. Additional optimization of the cell stimulation protocol and cell lysis procedure may be required for each specific application.
2. Remove the cellular debris by centrifuging the lysates at 14 000 x g for 10 minutes. Alternatively lysates may be ultracentrifuged at 100,000 x g for 30 minutes for greater clarification.
3. Carefully decant the clarified cell lysates into clean tubes and determine the protein concentration using a suitable method, such as the Bradford assay. Polypropylene tubes are recommended for storing cell lysates.
4. React an aliquot of the lysate with an equal volume of 2x Laemmli Sample Buffer (125 mM Tris, pH 6.8, 10% glycerol, 10% SDS, 0.006% bromophenol blue, and 130 mM dithiothreitol [DTT]) and boil the mixture for 90 seconds at 100°C.
5. Load 10-30 µg of the cell lysate into the wells of an appropriate single percentage or gradient minigel and resolve the proteins by SDS-PAGE.
6. In preparation for the Western transfer, cut a piece of PVDF slightly larger than the gel. Soak the membrane in methanol for 1 minute, then rinse with ddH_2O for 5 minutes. Alternatively, nitrocellulose may be used.
7. Soak the membrane, 2 pieces of Whatman paper, and Western apparatus sponges in transfer buffer (formulation provided below) for 2 minutes.
8. Assemble the gel and membrane into the sandwich apparatus.
9. Transfer the proteins at 140 mA for 60-90 minutes at room temperature.
10. Following the transfer, rinse the membrane with Tris buffered saline for 2 minutes.
11. Block the membrane with blocking buffer (formulation provided below) overnight at 4°C or for one hour at room temperature.
12. Incubate the blocked blot with primary antibody at a 1:1000 starting dilution in Tris buffered saline supplemented with 1% Ig-free BSA and 0.1% Tween-20 overnight at 4°C or for two hours at room temperature.
13. Wash the blot with several changes of Tris buffered saline supplemented with 0.1% Tween 20.
14. Detect the antibody band using an appropriate secondary antibody, such as goat $F(ab')_2$ anti-rabbit IgG alkaline phosphatase conjugate (Cat. # ALI4405) or goat $F(ab')_2$ anti-rabbit IgG horseradish peroxidase conjugate (Cat. # ALI4404) in conjunction with your chemiluminescence reagents and instrumentation.

Cell Lysis Buffer
Formulation:
10 mM Tris, pH 7.4
100 mM NaCl
1 mM EDTA
1 mM EGTA
1 mM NaF
20 mM $Na_4P_2O_7$
2 mM Na_3VO_4
0.1% SDS
0.5% sodium deoxycholate
1% Triton-X 100
10% glycerol
1 mM PMSF (made from a 0.3 M stock in DMSO) or 1 mM AEBSF (water soluble version of PMSF)
60 µg/mL aprotinin
10 µg/mL leupeptin
1 µg/mL pepstatin
(alternatively, protease inhibitor cocktail such as Sigma Cat. # P2714 may be used)

Transfer Buffer
Formulation:
2.4 gm Tris base
14.2 gm glycine
200 mL methanol
Q.S. to 1 liter, then add
1 mL 10% SDS.
Cool to 4°C prior to use.

Tris Buffered Saline
Formulation:
20 mM Tris-HCl, pH 7.4
0.9% NaCl

Blocking Buffer
Formulation:
100 mL Tris buffered saline
5 gm Ig-free BSA
0.1 mL Tween 20

Peptide Competition Experiment

BioSource's Phosphorylation Site Specific Antibodies (PSSAs) have been developed to enable the specific and sensitive detection of phosphorylation of particular amino acid residues in target proteins, while circumventing the need for protein purification, phosphopeptide mapping or handling radioactivity. The specificity of a PSSA in each experimental system can be confirmed through peptide competition. In this technique, aliquots of antibody are pre-incubated with peptide containing the sequence of the phosphopeptide immunogen used to raise the PSSA and the corresponding non-phosphopeptide. Following preincubation with the peptide, each antibody preparation is then used as a probe in antibody-based detection methods, such as Western blotting, immunocytochemistry, flow cytometry, or ELISA. With a PSSA specific for the phosphorylated target protein, pre-incubation with an excess of peptide containing the sequence of the phosphopeptide immunogen will block all antigen binding sites, while pre-incubation with the corresponding non-phosphopeptide will not affect the antibody.

BioSource has developed a line of control peptides specifically for use in peptide competition experiments with our PSSAs. These peptides, available as separate BioSource catalog items, are provided in pairs which contain the sequences of the phosphopeptide immunogen and the corresponding non-phosphopeptide.

In performing the Peptide Competition Experiment, it is important to note that the optimal dilutions of both antibody and peptide should be determined empirically for each specific application. The optimal dilution of antibody in these procedures is below saturating, as determined by previous experiments in your system. If an optimal antibody dilution has not been determined in your system, please refer to the Suggested Working Dilution on the antibody Product Analysis Sheet for guidance on an appropriate starting dilution. The optimal dilution of peptide used in these procedures will depend on the overall affinity or avidity of the antibody, as well as the quantity of the target antigen. A 50-150 fold molar excess of peptide to antibody is found to be effective for most peptide competition experiments.

In the example presented below, the PSSA is used as a dilution of 1:1000 and the peptides are used at a concentration of 333 nM. The total volume of the phosphopeptide and non-phosphopeptide-pre-incubated antibody preparations is 2 mL, sufficient for probing Western blot strips, as well as for use in other antibody-based detection methods. Under these conditions, the molar excess of peptide to antibody is ≥50.

Procedure:

1. Prepare three *identical test samples*, such as identical PVDF or nitrocellulose strips to which the protein of interest has been transferred. The test samples should be blocked using a blocking buffer, such as Tris buffered saline supplemented with 0.1% Tween 20, and either 5% BSA or 5% non-fat dried milk.
2. Prepare 6.5 mL of *working antibody stock solution* (1:1000 in this example) by adding 6.5 µL of antibody stock solution to 6.5 mL of buffer containing blocking protein, such as TBS supplemented with 0.1% Tween 20, and either 3% BSA or 3% non-fat dried milk.
3. Apportion the unused PSSA into working aliquots and store at –20°C for future use (the stock PSSA contains 50% glycerol and will not freeze at this temperature).
4. Allow the *lyophilized control peptides* to reach room temperature, ideally under desiccation.
5. Reconstitute each of the control peptides (supplied at 0.1 mg/vial) to a concentration of 66.7 µM with nanopure water. For a peptide with a molecular mass of 1500 (stated on the peptide Product Analysis Sheet), reconstitution with 1 mL water yields a solution with a concentration of 66.7 µM.
6. Apportion the unused reconstituted peptide solutions into working aliquots and store at -20°C for future use.
7. Label 3 test tubes as follows:
   - tube 1: water only no peptide control
   - tube 2: phosphopeptide
   - tube 3: non-phosphopeptide
8. Into each tube, pipette the following components
   - tube 1: 2 mL diluted PSSA solution plus 10 µL nanopure water
   - tube 2: 2 mL diluted PSSA solution plus 10 µL phosphopeptide
   - tube 3: 2 mL diluted PSSA solution plus 10 µL non-phosphopeptide
9. Incubate the three tubes for 30 minutes at room temperature with gentle rocking. During this incubation, the peptides have the chance to bind to the combining site of the antibody.
10. At the end of the incubation step, transfer the contents of each of the three tubes to clean reaction vessels containing one of the three identical test samples.

    For Western blotting strips:
    - Incubate the strips with the pre-incubated antibody preparations for 1 hour at room temperature or overnight at 4°C.
    - Wash each strip four times, five minutes each, to remove unbound antibody.
    - Transfer each strip to a new solution containing a labeled secondary antibody [e.g., goat $F(ab')_2$ anti-rabbit IgG alkaline phosphatase conjugate (Cat. # ALI4405) or goat $F(ab')_2$ anti-rabbit IgG horseradish peroxidase conjugate (Cat. # ALI4404)].
    - Remove unbound secondary antibody by thorough washing, and develop the signal using your chemiluminescent reagents and instrumentation.

The signals obtained with antibody incubated with the "Water Only, No Peptide Control" (Tube 1) represents the maximum signal in the assay. This signal should be eliminated by pre-incubation with the "Phosphopeptide" (Tube 2), while pre-incubation with the "Non-Phosphopeptide" (Tube 3) should not impact the signal. If the "Phosphopeptide" only partially eliminates the signal, repeat the procedure using twice the volume of water or peptide solutions listed in Step 8. If partial competition is seen following pre-incubation with the "Non-Phosphopeptide", repeat the procedure using half the volumes of water or peptide solutions listed in Step 8.